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The Plant Journal (2003) **35**, 763–770 doi: 10.1046/j.1365-313X.2003.01846.x

Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress

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Summary

Transgenic alfalfa root cultures expressing sense and antisense barley hemoglobin transcripts were examined under varying levels of atmospheric oxygen. Root cultures overexpressing the hemoglobin gene (Hb^+) maintained root growth when placed under 3% oxygen, whereas control cultures or cultures underexpressing hemoglobin (Hb^-) experienced 30–70% declines in growth under the same conditions. ATP levels and ATP/ADP ratios for Hb^+ lines did not significantly differ in 40 and 3% oxygen, whereas the ATP levels and ATP/ADP ratios in control and Hb^- lines were significantly lower under 3% oxygen. Large increases in the production of nitric oxide (NO) were measured in root cultures grown under hypoxic conditions compared to aerobic conditions. The amount of NO accumulated in an Hb^- line was 2.5-fold higher than that in the Hb^+ line. Treatment of transgenic root lines under 40% oxygen with NO resulted in significant declines in the ATP levels and ATP/ADP ratio of an Hb^- line and the control line, with no significant change in an Hb^+ line. The root cell structure of an Hb^- line showed evidence of cell breakdown under hypoxic growth, whereas an Hb^+ line had no evidence of cell breakdown under similar growth conditions. These results lead us to hypothesize that NO is involved in the response of plants to hypoxia and that hemoglobin modulates the levels of NO in the hypoxic cell.

Keywords: hypoxic stress, hemoglobin, nitric oxide, ATP, ATP/ADP, root growth.

Introduction

Hemoglobins are believed to be universally expressed amongst members of the plant kingdom (Andersson *et al.*, 1996; Arredondo-Peter *et al.*, 1998; Taylor *et al.*, 1994). One class of hemoglobins (Class 1) is induced in tissues exposed to hypoxic stress (Taylor *et al.*, 1994). Its induction is regulated by ATP or some consequence of ATP action (Nie and Hill, 1997). Cells expressing this stress-induced hemoglobin maintain their energy status under hypoxia more effectively than cells not expressing the gene (Sowa *et al.*, 1998).

Nitric oxide is a known metabolite in most biological systems. It is produced in animals during hypoxic stress (Bredt and Snyder, 1994), where it is involved in the regulation of blood vessel capillary dilation. In microbial systems, NO is detoxified through a reaction with a flavohemoglobin (Gardner *et al.*, 1998). NO is produced under a variety of conditions in plants (Durner and Klessig, 1999). There are suggestions that it acts as a signal molecule in plants (Durner and Klessig, 1999), as it does in animal systems (Ignarro, 1992). It has a regulatory role in apoptosis (Kim *et al.*, 2001) and it has been suggested that it may initiate programmed cell death in plant cells near invasion sites of pathogens (Richberg *et al.*, 1998).

Many plant roots adapt to flooding stress through the development of aerenchyma (reviewed by Drew, 1997), which permits the movement of air from the shoots via the generated channels. Aerenchyma formation occurs through selective cell death, although it is still unclear whether the process is programmed or is a result of cell necrosis. There is evidence that ethylene is involved in aerenchyma formation in some plants (Drew *et al.*, 1979), but the universality of this involvement has been questioned (Jackson, 1985). There are suggestions that ethylene generation may be regulated by NO (Haramaty and Leshem, 1997) and that NO may be involved in a signal amplification loop involving ethylene in the plant immune response (McDowell and Dangl, 2000).

It has not been determined whether the expression of Class 1 hemoglobin has an effect on plant growth and development under hypoxic conditions. Nor is it known whether NO is produced in plants under hypoxic stress and whether NO production has an effect on plant growth or development. Using barley hemoglobin cDNA (Taylor *et al.*, 1994), we have developed transgenic alfalfa root cultures that vary in the expression of hemoglobin to determine the effect of the transformations on root growth and development during hypoxia. We have also examined whether NO is produced in the roots under hypoxic conditions and whether changing the expression of hemoglobin has an effect on the levels of NO found.

Results

Effect of hemoglobin variation on alfalfa root growth

The variation in the hemoglobin content, under 3% oxygen of the transgenic lines selected for the study is shown in Table 1. There was about a 19-fold variation in hemoglobin content between the sense lines (Hb^+) and the antisense lines (Hb^-). The suppression of the expression of alfalfa hemoglobin by antisense barley hemoglobin varied between 65 and 90% in the two lines tested, confirming the close homology between the two hemoglobins (Serégelyes *et al.*, 2000). The absolute amounts of hemoglobin may be underestimated, as a barley hemoglobin antibody and barley hemoglobin have been used to estimate the native alfalfa hemoglobin. The relative amounts, however, should not be affected by the assay procedure as all lines are expressing the alfalfa protein. While the content of hemoglobin in the control (C) and Hb^- lines varied with the oxygen levels in the growth environment, the lines overexpressing barley hemoglobin (Hb^+) displayed the same levels of hemoglobin regardless of oxygen tension.

The root growth rates of the alfalfa lines at three different levels of oxygen tension were determined over a 3-day period (Figure 1). With the exception of line Hb^-(24) that had significantly higher root growth, there were no significant differences in the root growth rates of any of the lines grown at 40% oxygen. The control line, all of the Hb^- lines

Table 1 Hemoglobin content of transgenic alfalfa lines after exposure to 3% oxygen for 24 h

Alfalfa line	Hemoglobin content (nmol g^{-1} protein)
Control	6.3 ± 0.6
Hb^+(3)	9.4 ± 1.3
Hb^+(209)	11.8 ± 1.4
Hb^-(24)	2.2 ± 0.2
Hb^-(44)	0.64 ± 0.08

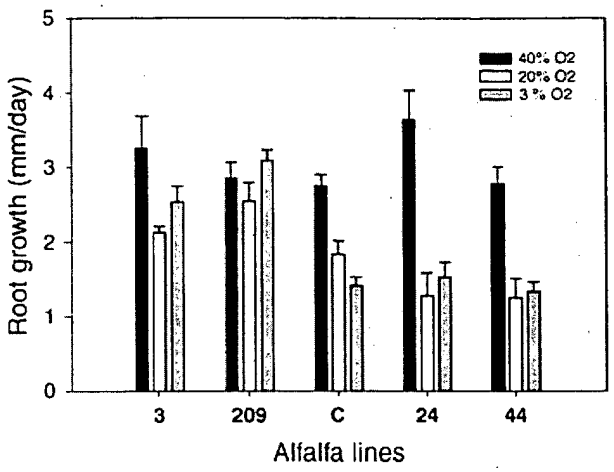

Figure 1. Effect of oxygen concentration on root growth of alfalfa root culture lines. Values are mean ± SE of six replicates and representative of three independent experiments. Lines 3 and 209 are Hb^+ lines, C is the control line and 24 and 44 are the Hb^- lines.

and one of the Hb^+ lines (3) showed significant declines in growth rate from 40 to 20% oxygen with the Hb^- lines showing the greatest percentage declines in growth rate. At 20% O_2, the Hb^+ lines had significantly higher growth rates than the control line which, in turn, had significantly higher growth rates than the Hb^- lines. Only the control line showed a significant decline in growth rate at 3% compared to 20% O_2. All other lines showed increases in growth rate, with the growth rate being significantly higher for Hb^+(3). There were no significant differences in the growth rates of the Hb^+ lines at 3% compared to 40% O_2, whereas there were large, significant decline in the growth rates of the control and Hb^- lines.

A relationship has been demonstrated between the expression of the hemoglobin gene and energy charge during hypoxia in maize cells (Sowa *et al.*, 1998). Under 40% O_2, the ATP levels in the Hb^- alfalfa root lines were not significantly different from the control line but were significantly lower than the Hb^+ lines (Figure 2a). Under 3% O_2, there were large significant declines in the ATP levels of the Hb^- lines compared to the 40% O_2 treatment and a smaller, but significant, decline in the ATP level of the control line. The Hb^+ line, when treated with 3% O_2, showed small, non-significant declines in ATP in comparison to 40% O_2. At 3% O_2, the Hb^+ lines had significantly higher ATP levels than the control line, which had significantly higher ATP levels than the Hb^- lines. The ATP/ADP ratios of the Hb^- lines under 40% O_2 were significantly lower than those of the control and Hb^+ lines (Figure 2b). When treated with 3% O_2, there were significant decline in the ATP/ADP ratios of the Hb^- and control lines, but no significant declines in the Hb^+ lines.

Measurement of alcohol dehydrogenase (ADH) activity was used as an indicator of the degree of hypoxic stress

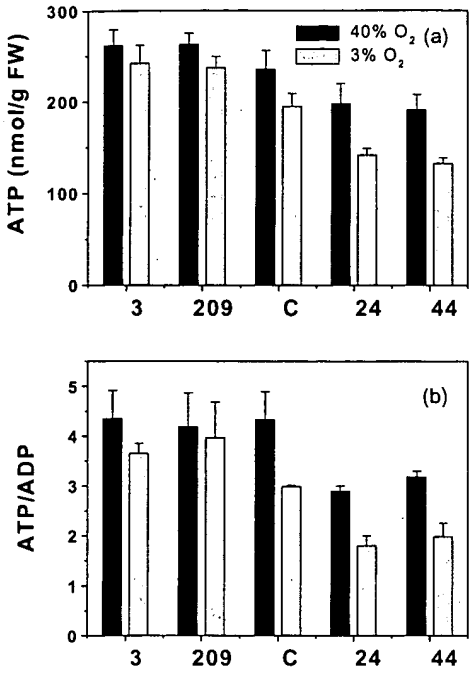

Figure 2. ATP levels (a) and ATP/ADP ratios (b) in roots of Hb^+(3, 209), control (C), and Hb^-(24, 44) alfalfa root cultures exposed to 3 and 40% O_2.

imposed on the root lines (Figure 3). Alcohol dehydrogenase activity increased significantly in each line as the oxygen levels decreased from 40–20 to 3%. The largest differences in ADH activity occurred between the 20 and 3% oxygen treatments. While there were significant differences between the various lines at each oxygen concentration, there was no distinct trend. At the 3% oxygen level, the ADH activity increased inversely to the hemoglobin levels, with Hb^- lines having a significantly higher ADH activity than the control or Hb^+ line.

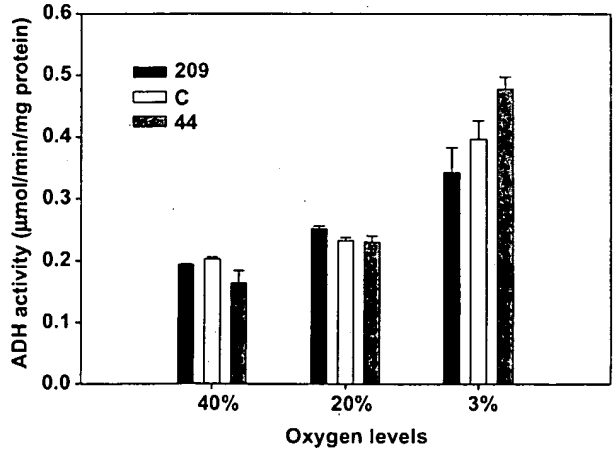

Figure 3. ADH-specific activity in roots of Hb^+(209), control (C), and Hb^-(44) alfalfa root culture lines exposed to 3, 20, and 40% O_2.

Microscopic examination of cross-sections of an Hb^+ and an Hb^- line (Figure 4) demonstrated differential effects upon treatment with 40 and 3% oxygen over a 5-day period. The changes in the cell structure of the Hb^+ line as the oxygen concentration was changed from 40 to 3% oxygen were slight, at best. There was evidence of cell disruption in the 3% oxygen treatment in the control line. In the Hb^- line, however, there was strong evidence of cell disruption in the 3% oxygen treatment when compared with the Hb^-, 40% oxygen treatment or the Hb^+, 3% oxygen treatment. Longitudinal sections of the two lines showed that the root tips remained relatively unchanged, whereas the cells distal to the root tip showed evidence of cell disruption in the Hb^- line (data not shown).

Nitric oxide in alfalfa roots

The production of NO was measured in Hb^+, control, and Hb^- lines under 3 and 40% oxygen by measuring, using EPR spectroscopy, the total NO trapped (Figure 5). Over a period under high oxygen tension, none of the lines produced significant NO. However, under low oxygen tension a substantial amount of NO was trapped. The amount of trapped NO varied in an inverse relationship to the level of hemoglobin in the root line, with significant differences in NO between each line. About 2.5-fold more NO was trapped in the medium of the Hb^- line than in the Hb^+ line. Figure 6 shows the distribution of NO along the first 3 cm of a control root under 3% oxygen. There is little NO in the root tip. The distribution in the 3 cm from the tip is uniform and about seven times the levels found in the tip.

To determine whether the added NO had a differential effect on adenine nucleotide levels of lines varying in Hb expression, an Hb^+, a control, and an Hb^- line were treated with an NO donor (3 μM *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP; Alexis Biochemicals, San Diego, CA, USA)) and the effect on ATP levels and ATP/ADP ratios was determined (Figure 7). SNAP significantly decreased the ATP levels (Figure 7a) and ATP/ADP ratios (Figure 7b) of the Hb^- and control lines, respectively, but not that of the Hb^+ line.

Discussion

Earlier work has demonstrated that hemoglobin acts to maintain the energy status of plant cells exposed to low oxygen tensions (Sowa *et al.*, 1998). The results in Figure 2 indicate that this is also true for alfalfa root lines. As cell energy status is an indicator of cell growth status (Atkinson, 1965), maintenance of energy levels under hypoxia should result in the maintenance of growth. The present study demonstrates a relationship between constitutive hemoglobin expression and the maintenance of root growth rate under hypoxic conditions. Root growth rates of transgenic

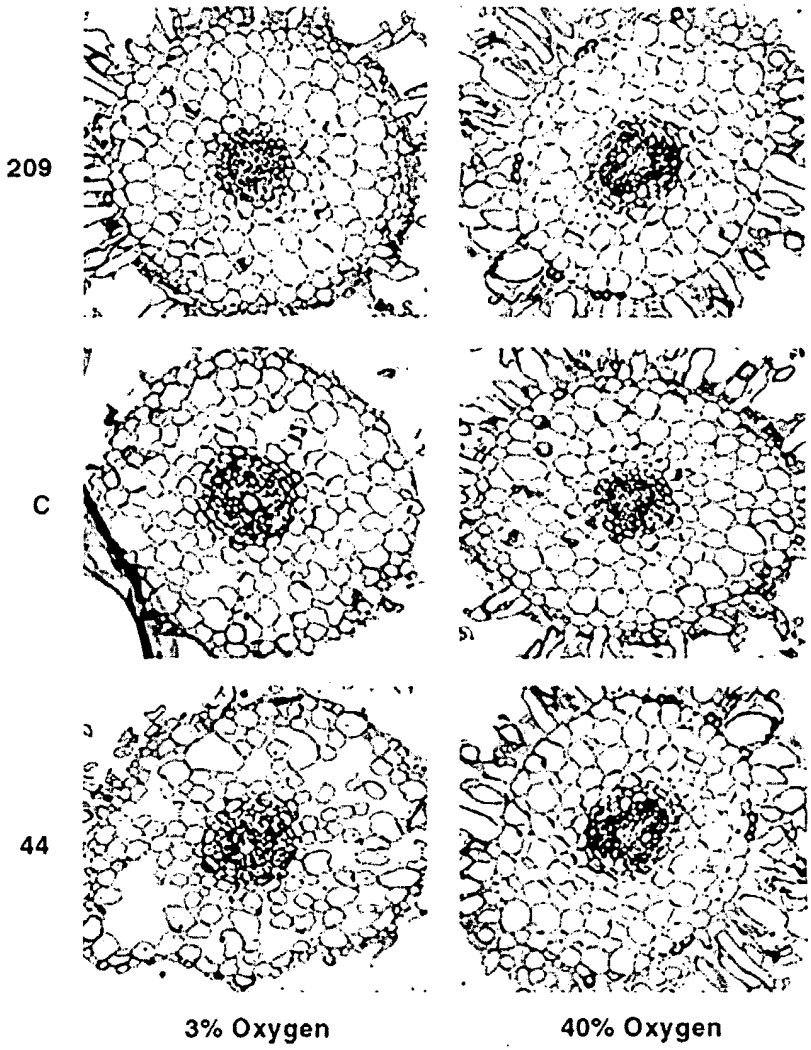

Figure 4. Root cross-sections of Hb^+(209), control (C) and Hb^-(44) alfalfa root culture lines after treatment with 3 or 40% oxygen over a 5-day period. Root segments of about 2–3 cm were exposed to 3 and 40% oxygen and then were fixed, dehydrated, infiltrated and embedded in JB-4 resin. The embedded tissue was sectioned 2 cm behind the root tip in 3 μm sections and stained with toluene blue.

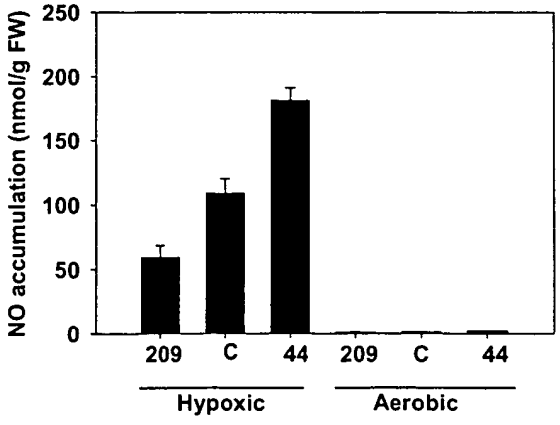

Figure 5. Accumulation of NO in Hb^+(209), control (C), and Hb^- (44) alfalfa root culture lines grown under hypoxic and aerobic conditions for 24 h.

alfalfa lines overexpressing hemoglobin maintained higher growth rates under normal or reduced oxygen tensions than those of either transgenic lines, in which alfalfa hemoglobin expression was suppressed or the untransformed parent (Figure 1). Similar effects on root growth have been demonstrated for transgenic *Arabidopsis*, transformed to express varying levels of hemoglobin (Hunt *et al.*, 2002).

Roots are the most sensitive organ to hypoxic stress in vascular plants. Indeed, plants subjected to waterlogging often exhibit a dramatic reduction in root growth (Trought and Drew, 1980; Waters *et al.*, 1991). This reduced growth has been attributed to declines in the level of ATP in anoxic roots (Atwell *et al.*, 1982). Studies of ATP levels in anoxic roots have shown that ATP levels can be reduced to less than 5% of the levels in aerobic roots (Drew *et al.*, 1985). Our results showing that alfalfa roots expressing hemoglobin maintain ATP levels and root growth under low oxygen tensions, in conjunction with the evidence that hemoglobin expression maintains cell energy status, (Sowa *et al.*, 1998)

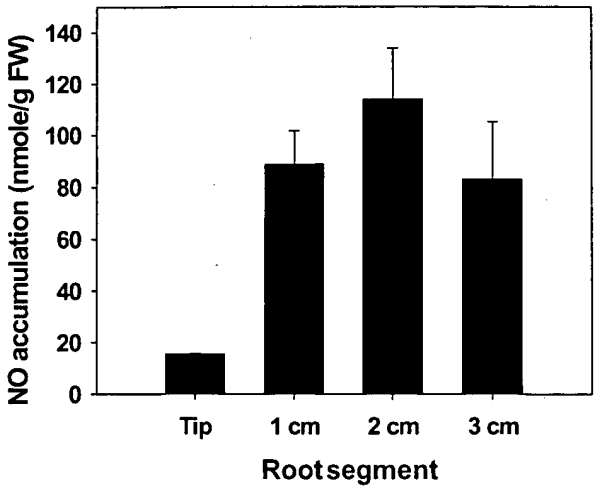

Figure 6. NO distribution along the length of alfalfa control (C) roots exposed to hypoxic conditions. Root segments of 2 mm (tip), 1–3 cm (measured from the tip of the root) were cut, placed in flasks containing MSHF medium for 24 h under 3% oxygen and the levels of NO in the tissue were measured by EPR.

indicate a strong relationship between hemoglobin, energy status and the maintenance of root growth under hypoxic conditions. The declines observed in the ATP levels and ATP/ADP ratios in Hb^- lines when NO is supplied externally

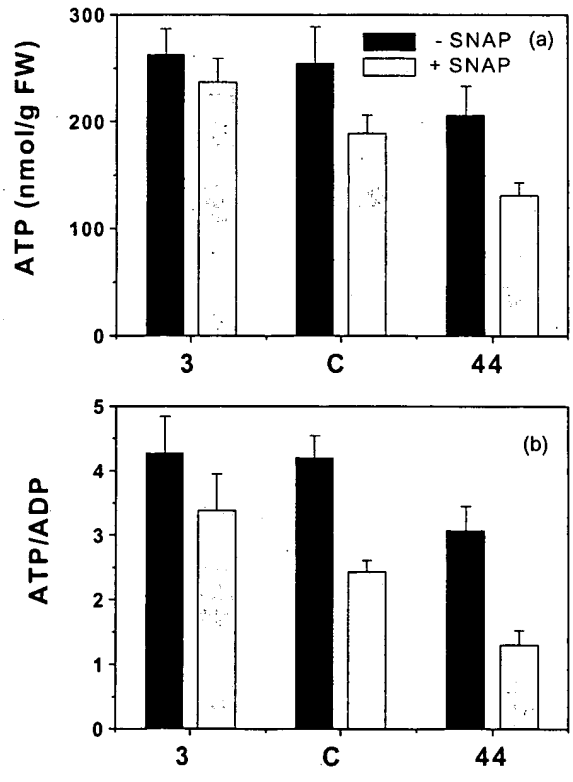

Figure 7. Effect of externally applied NO on the ATP levels (a) and ATP/ADP ratios (b) of transgenic alfalfa roots under 40% oxygen. The lines used were Hb^+(3), control (C), and Hb^-(44).

(Figure 7) are consistent with such a relationship and suggest that a possible mechanism by which hemoglobin acts is through modulation of NO levels.

Transgenic alfalfa overexpressing hemoglobin showed no evidence of change in cell structure in roots exposed to low oxygen tensions (Figure 4). Roots of hemoglobin-underexpressing lines, on the other hand, demonstrated cellular disintegration characteristic of cell death. NO is known to be involved in programmed cell death in mammalian systems (Kim *et al.*, 2001) and has been recognized as a potential agent in programmed cell death in plants (Richberg *et al.*, 1998). It has been shown to induce cell death in *Arabidopsis* suspension cultures, possibly through the activation of guanylate cyclase (Clarke *et al.*, 2000). Hb^+ lines have lower NO levels and show no evidence of cellular disintegration under hypoxic conditions, whereas Hb^- lines have high NO levels and show evidence characteristic of cell death under these same conditions (Figures 4 and 5), suggesting that NO may be involved in cell death. Control roots under 3% oxygen showed uniform levels of NO in regions removed from the root tip (Figure 6). This observation in conjunction with the evidence that aerenchyma formation is known to involve cell death and does not occur in the root tip (Drew *et al.*, 1979) provides additional support for the relationship between NO and root cell death during hypoxia.

There is a consistent relationship between the hypoxic stress response relative to hemoglobin expression in the tissue. The Hb^- line, with the highest ADH activity (Figure 3), has the greatest appearance of cell death (Figure 4), while the Hb^+ line has the lowest ADH activity and no apparent cellular disintegration. This follows through with the ATP and ATP/ADP values (Figure 2), where Hb^+ lines show the least stress (high ATP, high ATP/ADP) and Hb^- lines show the greatest stress (low ATP, low ATP/ADP). Similar relationships of ADH activity and ATP levels in Hb over- and under-expressing maize cell lines in 3% O_2 have been observed (Sowa *et al.*, 1998).

Nitric oxide and hemoglobin are intimately linked in many biological systems (Durner and Klessig, 1999; Poole and Hughes, 2000; Weber and Vinogradov, 2001) and their interaction is frequently associated with a response to low oxygen tension. Our results (Figure 5) demonstrate that plants also respond to low oxygen tensions with production of NO. Recent work (Flogel *et al.*, 2001) has indicated that myoglobin may act as a scavenger of NO in muscle. While the mechanisms by which NO and hemoglobin interact and function in plants have not been elucidated, studies in other systems do provide potential plant pathways that can be examined. In the presence of oxyhemoglobin or oxymyoglobin, NO is rapidly oxidized to nitrate (Ignarro *et al.*, 1993). The variation in NO levels in alfalfa root lines differing in their hemoglobin protein expression strongly suggest that hemoglobin may be

effecting turnover of NO by reaction with oxyhemoglobin to form nitrate.

We have proposed a hypothesis for the role of hemoglobin in plants experiencing low oxygen tensions that involves NO (Dordas *et al.*, 2003) and concentrates on the metabolic aspects of hemoglobin function. The present study examined the relationship between hemoglobin and alfalfa root growth responses under hypoxic conditions and provided evidence to suggest that one major function of hemoglobin may be to modulate NO levels in the plant cell.

Experimental procedures

For the transformation experiment, alfalfa (*Medicago sativa* cv. Regen SY) was used. It is highly regenerable and has been extensively used in the past for transformation experiments (McKersie *et al.*, 1993). Alfalfa seeds were scarified and sterilized with ethanol and commercial bleach (one-fourth strength). The seeds were placed in magenta boxes containing MS media without hormones (MSHF) (Murashige and Skooge, 1962). Plants were propagated by transferring 5-cm-long shoots with a few internodes to new MS media every 4 weeks under aseptic conditions. Two strains of *Agrobacterium rhizogenesis*, A4 and 8196, were tested using two different orientations of the alfalfa stem: shoot-end upright and shoot-end inverted. Strain A4 was superior at producing transgenic roots, as was the shoot-end inverted orientation (data not shown).

Construction of transformation vectors

Constructs containing the sense and antisense orientation of barley hemoglobin were obtained from pAS1 (containing sense) or pAS2 (containing antisense) plasmids (Sowa *et al.*, 1998). The fragment containing ubiquitin promoter + ubiquitin intron + hemoglobin + nos3′ was inserted into the vector pWBVec8 (Wang *et al.*, 1998). The plasmids were used to transform *A. rhizogenesis* strain A4 using the freeze-thaw method (An *et al.*, 1988). After the transformation, the bacteria were spread onto a mannitol 8 g l^{-1}, yeast extract 5 g l^{-1}, casamino acids 0.5 g l^{-1}, ammonium sulfate 2 g l^{-1}, NaCl 5 g l^{-1}, agar 15 g l^{-1}, pH 6.6 (MYA) plate (Petit and Tempé, 1978) containing 100 μg ml^{-1} spectinomycin and incubated at 28°C for 3 days. Single colonies were collected and amplified in 20 ml of MYA containing 100 μg ml^{-1} spectinomycin at 28°C for 3 days. Plasmids were extracted from the bacteria and were restriction enzyme-digested to verify the transformation. The desired cells were amplified and stored at −75°C in the presence of 20% glycerol or on MYA plates containing spectinomycin.

Alfalfa transformation

Stem segments (2-cm long) were cut from alfalfa plants and placed inverted into Magenta boxes containing MSHF media (D'Halluin *et al.*, 1990). A loopful of *A. rhizogenes* A4 containing the appropriate constructs was placed on the exposed end of the explant. The control line (C) was transformed with an empty cassette. After a few weeks, one root from each stem segment was taken and placed on a Petri plate with MSHF media containing 500 mg l^{-1} carbenicillin and 20 mg l^{-1} hygromycin. Each root was left to grow for few weeks and then it was screened at the DNA and protein levels for insertion of the hemoglobin gene and for quantification of the levels of expression of hemoglobin. We selected two overexpressing lines, Hb^{+}(3) and Hb^{+}(209), with the highest levels of Hb expression and two underexpressing lines Hb^{-}(24) and Hb^{-}(44), with the lowest levels of Hb expression. These lines, along with the control line, were used for most of the experiments described in this study.

Protein immunoblotting

Roots (100 mg) were ground in liquid nitrogen with a mortar and pestle, and extracted with 0.2 ml of ice-cold extraction buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The protein content of the extract was measured (Bradford, 1976) with BSA as a standard. SDS-loading buffer (0.05 M Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 3% β-mercaptoethanol, and a trace of bromophenol blue) was added, the samples were boiled for 10 min and the proteins separated by SDS–PAGE using a Bio-Rad Miniprotean II gel apparatus according to an established protocol (Bio-Rad bulletin 1721). Final acrylamide concentration was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes and were detected using a polyclonal antibody raised against barley-recombinant hemoglobin. The protein concentration in different lines was calculated by densitometric comparison using Multi-Analyst PC software (Bio-Rad Laboratories, Mississauga, ON, USA) of immunoblots and recombinant barley Hb as a standard.

Growth measurements under different levels of O_2

In order to determine the effect of the Hb content in maintaining the root growth in alfalfa, we measured the growth of alfalfa root tips at three different levels of O_2 (3, 20, and 40%) to represent, respectively, hypoxia, normal atmospheric oxygen with reduced respiration, and an oxygen pressure in excess of the critical oxygen pressure (Saglio *et al.*, 1984). Root tips were placed on Petri plates containing MSHF media and placed in containers. The containers were flushed with either nitrogen containing 3 or 40% O_2 or air (20% O_2). Growth was measured daily with a ruler, over a period of 3 days. Each experiment was repeated three times with at least 10 root tips.

ATP and ADP measurements

Root sections (approximately 1 cm from tip) were placed into 30 ml syringes with continuous bubbling of 40% O_2 (for normoxic conditions) for 24 h. For hypoxic treatments, the gas mixture was then switched to 3% O_2 for 24 h. To produce an NO concentration of 1.5–2 μM in the medium (Zhang *et al.*, 2000), 3 μM SNAP was added to the medium, through which 40% oxygen was bubbled.

The roots (100 mg fresh tissue) were homogenized with a mortar and pestle under liquid N_2 with 0.5 ml of 10% perchloric acid. The homogenate was transferred into a microcentrifuge tube and the mortar and pestle was rinsed with 0.5 ml of perchloric acid which was then added to the original homogenate. The extract was neutralized with a solution of 5 M KOH and 1 M triethanolamine using pH paper to estimate neutral pH. The resultant salt precipitate was removed by centrifugation (16 000 *g*), the supernatant was then transferred into a new tube and the final volume was monitored. All operations were performed at 4°C.

The concentrations of adenine nucleotides were measured by the firefly luciferase method (Holm-Hansen and Karl, 1978) using a portable luminometer (Model Junior LB 9509, Berthold

Technologies, Bad Wildbad, Germany). ADP was converted to ATP using pyruvate kinase (Roche Diagnostics, Laval, Canada).

Assay of alcohol dehydrogenase activity

The activity of ADH was measured according to Hanson *et al.* (1984). Fresh tissue (100 mg) was ground in a mortar and pestle with liquid nitrogen and extracted with buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM DTT, 20% glycerol. The extracts were centrifuged at 10 000 *g* for 20 min at 4°C and the supernatant was assayed for ADH activity and protein content. The ADH assay buffer contained 0.1 M glycine NaOH (pH 9), 0.1 M ethanol, and 2 mM NAD. Protein was determined (Bradford, 1976) using BSA as a standard.

Anatomical studies

Root segments were grown on Petri plates containing MSHF media and exposed to different concentrations of O_2 (3 and 40%). After 5 days, the roots were collected from the Petri plate and were fixed overnight in 3% glutaraldehyde in 50 mM phosphate buffer. The tissue was dehydrated in an ethanol series, infiltrated with JB-4 infiltration solution (Emsdiasum, Fort Washington, PA, USA) overnight and was embedded in the JB-4 resin. The embedded tissue was sectioned 1–2 cm behind the root tip in the 3-μm sections and stained with toluene blue. The sections were viewed with a Zeiss microscope and the images were recorded with a Nikon (Mississauga, ON, USA) digital camera.

Hypoxic treatment of alfalfa roots and determination of nitric oxide production using EPR spectroscopy

Root tips (1 cm in length) were collected, and about 0.1 g of tissue was used for the experiment in 1 ml of MSHF media. For the hypoxic treatment, the flasks were closed with serum caps and were flushed with N_2 via a ventilation needle for 2 min. For aerobic treatment, the flasks were covered with aluminum foil to allow exchange of oxygen. Flasks were placed on a shaker at a speed of 150 r.p.m. All treatments were performed under sterile conditions, in the presence of 500 mg l^{-1} carbenicillin to inhibit bacterial growth. The production of NO in root sections was determined by taking sections of root tip (about 2 mm of the root) and 1-cm sections of root measured back from the root tip. All hypoxic treatments were for 24 h.

The NO spin trap Fe^{2+}-$(MGD)_2$ was used to measure NO production by electron paramagnetic resonance (EPR) spectroscopy. The Fe^{2+}-$(MGD)_2$ complex was prepared immediately before each experiment by mixing *N*-(dithiocarbamoyl)-*N*-methyl-D-glucamine (MGD; Alexis Biochemicals, San Diego, CA, USA) with fresh $FeSO_4$. Briefly, 500 μl of a 200 mM solution of MGD prepared in 0.1 M Hepes (pH 7.0) buffer was added to 500 μl of 10 mM $FeSO_4$ in 1 mM H_2SO_4. After a few minutes, when the reaction was complete, 100 μl of this solution was added to the treatment flasks to give final concentrations of 10 mM MGD and 0.5 mM $FeSO_4$. After the incubations, 15 μl of the treatment flask solutions were placed in glass capillary tubes for EPR analysis. NO reacts with the spin-trap complex to produce a stable EPR-detectable Fe^{2+}-$(MGD)_2NO$ spin adduct with a characteristic three-line EPR spectrum (hyperfine coupling constant A_N = 12.5 G and g_{iso} = 2.04) (Kotake, 1996). The EPR spectra were recorded at room temperature on a Bruker EMX spectrometer (Billerica, MA, USA). A total of 10 spectra were recorded over a period of 8 min and their signals were averaged. The instrument settings were as follows: microwave power 20 mW, modulation amplitude 4 G, modulation frequency 100 kHz, microwave frequency 9.243 GHz, receiver gain of 2×10^4 and magnetic field centered at 3255 G with a 100 G scan range. The g_{iso}-value of 2.04 was calculated with reference to a 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma, Milwaukee, WI, USA) standard with a *g*-value of 2.0036 recorded at the same time (Henry *et al.*, 1991). NO was quantified by measuring the peak-to-peak signal height of the low field EPR signal of Fe^{2+}-$(MGD)_2NO$ in comparison to NO spin trapped from rapidly produced by 2-(*N,N*-diethylamino)-diazenolate-2-oxide (DEANO; Alexis Biochemicals). The detection limit was 0.5 μM NO.

Acknowledgements

We thank Doug Durnin for technical assistance. The work was supported by the Natural Sciences and Engineering Research Council of Canada (Grant OGP4689) (R.D.H.), Agriculture Research and Development Initiative (Manitoba) (R.D.H.), and the Canadian Institutes of Health Research (B.B.H.).

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Acta Biologica Hungarica 54 (1), pp. 15–25 (2003)

PHYTOGLOBINS AND NITRIC OXIDE: NEW PARTNERS IN AN OLD SIGNALLING SYSTEM IN PLANTS

Review

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(Received: May 5, 2002; accepted: June 5, 2002)

The recent review summarizes the major achievements in discovery of role of phytoglobins in mediation of nitric oxide generated cellular functions in higher plants. Genes encoding non-symbiotic hemoglobins have been cloned from several plant species. The expression pattern of these genes show tissue-specificity that is also under the control of stress factors like hypoxia. The nitric oxide has pivotal role in signalling pathway specifically in hypersensitive reactions and programmed cell death. Production of transgenic tobacco plants overexpressing the alfalfa hemoglobin showed altered necrotic symptoms after treatment with nitric oxide generating compounds or infection by necrotic pathogens. The present review helps to outline the similar relation between hemoglobin and nitric oxide in plants as it was found in animal cells.

Keywords: Hypoxia – germination – pathogens – salicylic acid – reactive oxygen species

INTRODUCTION

The discovery of plant non-symbiotic hemoglobins, or "phytoglobins" [4, 9], came significantly later than that of symbiotic hemoglobins [31]. Although the function of the symbiotic hemoglobins had long been known [8] by that time, of course the appearance of novel plant hemoglobin types raised many new questions, and by far not all of them have been answered yet. The shortness of time that has passed since then is of course just one reason, and maybe not the most important one. There are new results in other, but related fields, the appearance of which was necessary for asking those questions that can take us closer to the understanding the role of these proteins.

The tight oxygen-binding properties of non-symbiotic hemoglobins were the first signs to show a function which was quite different from what symbiotic hemoglobins were good for [6]. Inducibility by hypoxia and evidence for barley hemoglobin to take part in ATP metabolism also supported the distinct function of non-symbiotic hemoglobins [51]. Another milestone was to discover the importance of NO-binding by hemoglobins in animals [20]. The investigation of the possible functions of NO

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0236-5383/2003/$ 20.00

in plants has led to new and interesting results recently. These results suggested that many functions of NO in plants are very similar to its function in animals [23, 60]. Furthermore, it has been shown that not only animal hemoglobins, but also hemoglobins of unicellular organisms can interact with NO in a physiologically significant way [22, 38]. The interaction of hemoglobins with NO in a wide range of organisms from unicellulars to animals led to the investigation of such a relation *in planta*. These efforts using transgenic plants overexpressing non-symbiotic hemoglobins have brought remarkable results recently.

General remarks on hemoglobins

Hemoglobins are common to be found in various organisms ranging from unicellulars to higher plants and animals [3, 19, 44, 61]. The hemoglobins of higher organisms are either monomeric (e.g. leghemoglobin, [3]) or multimeric proteins, such as mammalian myoglobin and hemoglobin [61] and some plant non-symbiotic hemoglobins [25].

It is well known that animal hemoglobins bind and carry the gases of respiration, mainly O_2 and CO_2, and this is considered to be their main function. Plant hemoglobins can also bind these gases, and they are usually divided into three major groups on the basis of their common features.

Plant hemoglobins

The first group is formed by symbiotic plant hemoglobins. They can be found in leguminous plants and non-legumes living in symbiosis with nitrogen-fixing organisms. Their role is to provide oxygen to symbionts in tissues actively fixing nitrogen [2].

Non-symbiotic plant hemoglobins, termed also as "phytoglobins" [16], belong to the second group. They are not only present in plants containing symbiotic hemoglobins [1, 2, 4, 12, 34], but also in other plant species such as *Arabidopsis* [56], barley [55], rice [5], *Trema tomentosa* [9], alfalfa [48], etc. Furthermore, symbiotic hemoglobins are assumed to have evolved from non-symbiotic hemoglobins by gene duplication [34]. That is why non-symbiotic hemoglobins are considered not only to be more widespread, but also more ancestral than symbiotic hemoglobins [1]. This gave a basis to call non-symbiotic hemoglobins as phytoglobins, while other plant hemoglobins with specific function and location could be defined with the appropriate adjective or suffix [16]. Because the group of non-symbiotic hemoglobins has only been discovered relatively recently [4, 9], their function is not yet fully understood.

In general, the members of this group have much higher affinity for oxygen compared to symbiotic hemoglobins [6, 17] and are induced in plants under low oxygen tensions, which attributed an oxygen sensing function to phytoglobins [55, 56].

Hemoglobin genes from various plants (e.g. from *Brassica*, *Gossypium* and *Arabidopsis thaliana*) with close homology to non-symbiotic hemoglobins were shown to be induced by cold stress, not by hypoxia [56]. This gave the basis for the classification of non-symbiotic hemoglobins. According to Trevaskis et al., to class 1 those hemoglobin genes belong that are induced upon hypoxic treatment (e.g. *Mhb1* from alfalfa, *AHB1* from *Arabidopsis thaliana,* a non-symbiotic hemoglobin gene from barley, etc.). The cold-inducible non-symbiotic hemoglobin genes belong to class 2 [56].

Actually, it is not only hypoxia where class 1 genes have a role. For example the class 1 *AHB1* gene of *Arabidopsis thaliana* could also be induced by nitrate [58]. Furthermore, 2,4-dinitrophenol, a respiratory chain uncoupler, was shown to increase both oxygen consumption and barley hemoglobin expression in barley aleurone tissue. This indicated that the expression of barley hemoglobin is influenced by the availability of ATP in tissues. [42].

Barley non-symbiotic hemoglobin was also shown to be involved in ATP metabolism under hypoxia. It was observed that the ATP levels of a maize suspension culture overexpressing barley hemoglobin were about 30% higher than that of non-transformed maize cells, both grown under hypoxic conditions. On the other hand, the ATP content of maize suspension cells containing an antisense barley hemoglobin construct was about 30% less than that of the non-transformed maize cells when both cultures were grown under hypoxia [51].

High mRNA levels of phytoglobin were observed in the roots and rosette leaves of barley [55], young leaves, stems and roots of soybean [1], in rice leaves and roots [5], in *Arabidopsis thaliana* roots [56] and in alfalfa roots [48]. The accumulation of phytoglobin mRNA under non-hypoxic conditions is believed to occur because of the high metabolic activity of the above-mentioned tissues [1].

The third major group involves 2-on-2 plant hemoglobins. Their name implies a structural difference from the other two major groups, and their function is also thought to be different. Although they have some similarity to non-symbiotic hemoglobins, they have unique biochemical properties and evolutionary history. They show the highest homology to the truncated hemoglobins of microorganisms. Such a gene was found in *Arabidopsis thaliana* roots and shoots (*AHB3*), and shown to be down regulated by hypoxia [59].

The phylogram in Figure 1 shows the relation of the above-mentioned three major plant hemoglobin types to each other. The group of non-symbiotic hemoglobins is not only separated to class 1 and class 2 subgroups in the phylogram, but also the class 1 group is further divided to class 1 phytoglobins of dicots and those of monocots. This may indicate a functional difference between them in spite of their close homology. Furthermore, the class 2 phytoglobins show a closer relation to symbiotic hemoglobins than to their class 1 counterparts. The 2-on-2 type hemoglobin, AHB3 is also located on the branch of symbiotic hemoglobins, though it seems to be rather distantly related to them. The hemoglobins from *Trema* and *Parasponia* are separated from the major branches. This can be explained by their unique feature that they can fulfil both symbiotic non-symbiotic hemoglobin functions [9].

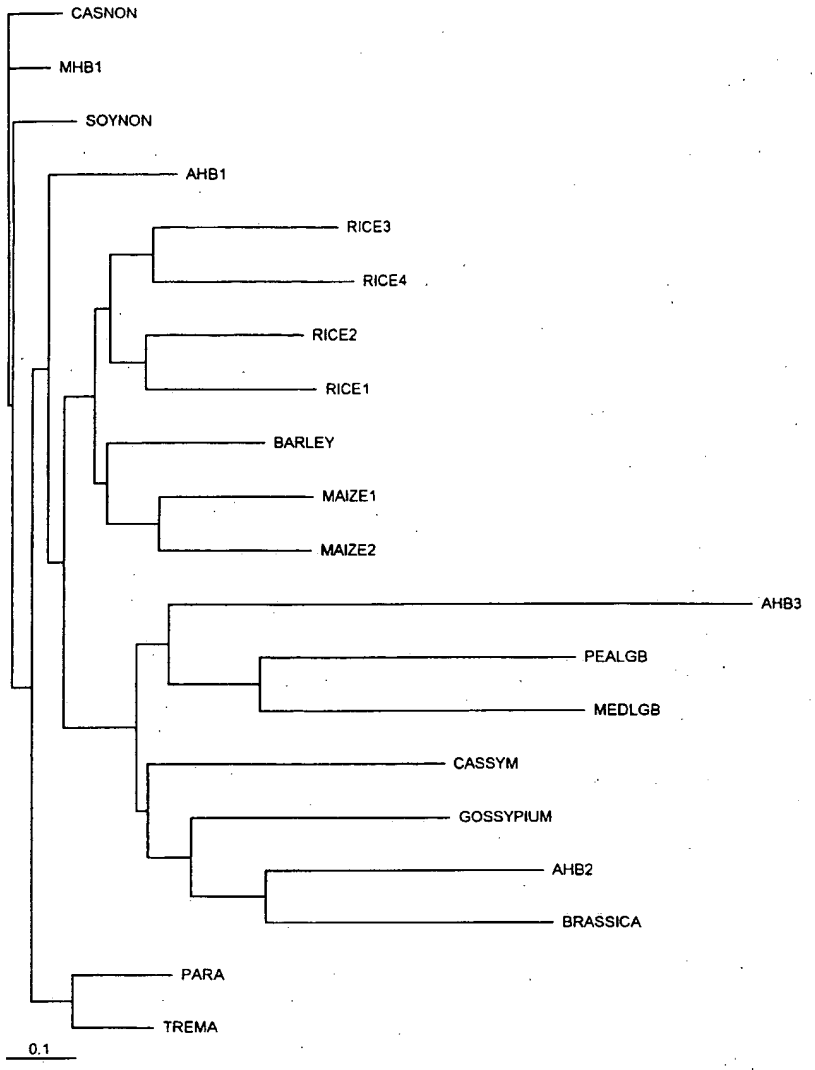

Fig. 1. Phylogram of some plant hemoglobins. Hemoglobins (with GenBank accession numbers or with references): MHB1: (AF172172), CASNON: *Casuarina glauca* non-symbiotic hemoglobin (X53950), SOYNON: soybean non-symbiotic hemoglobin (U47143), TREMA: *Trema tomentosa* non-symbiotic hemoglobin (Y00296), PARA: *Parasponia andersonii* hemoglobin (U27194), AHB1: *Arabidopsis thaliana* class1 non-symbiotic hemoglobin (U94998), MAIZE1 and MAIZE2: maize class 1 non-symbiotic hemoglobins (AAG01375 and AAG01183, respectively), BARLEY: barley non-symbiotic hemoglobin (U01228), RICE1, RICE2, RICE 3 and RICE4: rice class 1 non-symbiotic hemoglobins (U76029, U76028, AAM19124 and AAM19123, respectively), BRASSICA: *Brassica napus* class 2 non-symbiotic hemoglobin (AAK07741), GOSSYPIUM: *Gossypium hirsutum* class 2 non-symbiotic hemoglobin (AAK21604), AHB2: *Arabidopsis thaliana* class 2 non-symbiotic hemoglobin (U94999), CASSYM: *Casuarina glauca* symbiotic hemoglobin [30], MEDLGB: *Medicago sativa* class 1 leg-hemoglobin (X13375), PEALGB: pea leghemoglobin (AB015720)

Recently discovered roles of hemoglobin

In unicellular organisms and animals it has been demonstrated that most hemoglobin proteins interact with NO in some physiologically important manner. The study of this interaction led to the recent discovery that the role of hemoglobin in these organisms is not restricted to the simple molecule-carrier function in respiration. Mammalian hemoglobin interacts with NO either to form S-nitroso- (when NO is bound to cysteine β_{93}) or nitrosylhemoglobin (here NO is bound to heme) in the arterioles. Then, on entering the lung, hemoglobin undergoes an allosteric transition (from T to R conformation) induced by oxygen, during which all the NO groups are transferred from hemes to cysteine β_{93}. This molecule, the S-nitroso-oxyhemoglobin (with NO bound to thiol and O_2 to heme), enters the arterial circuit. When it reaches the arterioles and capilleries again, low oxygen tension induces the allosteric transition back to the T state. At the same time the NO is released from cysteine β_{93}. Since NO has a vasodilatatory effect, it dilates blood vessels and thereby facilitates O_2 delivery [20, 21, 54].

However, NO can interact with hemoglobin not only in the mammals. In the nematode, *Ascaris*, hemoglobin is thought to act as a deoxygenase, using NO to detoxify oxygen in this aerophobic organism [38]. In bacteria, the flavohemoglobins are thought to act as dioxygenases using O_2 to detoxify NO in order to avoid nitrosative stress [22].

Nitric oxide formation in animals and plants

In animal cells, the biosynthesis of NO is primarily catalyzed by different isoforms of the enzyme nitric oxide synthase (NOS) [40]. NOSs can oxidize L-arginine to L-citrulline and NO. NOS-like activity, based on the formation from L-arginine to L-citrulline or on the sensitivity to mammalian NOS inhibitors has been detected in several plants, but no plant *NOS* gene has been identified yet [14, 46]. NO is also produced enzymatically from NO_3^- in plants by the NAD(P)H-dependent nitrate reductase. Furthermore, in plants, non-enzymatic NO-formation is also possible as a result of nitrous oxide decomposition and of chemical reaction of nitrite at acidic pH [60].

NO-related effects in plants

Multiple and important effects and functions of NO have been discovered in plants recently. Some of them coincide with NO functions already described in animals. For example in animals, NO can function as a messenger involved in several pathophysiological processes including programmed cell death [37] and those of immune, nervous and vascular systems [47, 57]. The NO signalling in animals can either be cyclic guanosine monophosphate (cGMP)-dependent or- independent [22]. In the cGMP-dependent pathway NO activates guanylate cyclase and the cell's cGMP level is

increased. Here cGMP is involved in smooth muscle relaxation, inhibition of platelet aggregation and in sensory systems [47].

NO has cGMP-independent biological effects in animals in the form of peroxynitrite ($ONOO^-$). Peroxynitrite is formed from NO and the NADPH oxidase product superoxide (O_2^-), and is believed to play a role in apoptosis in animals [7]. The situation of peroxynitrite formation is similar in plant defense responses [60], but its importance was shown to be less in the hypersensitive reaction (HR) than in animal cells [15].

Another cGMP-independent reaction is nitrosylation, which can modify signal transduction. There are NO-responsive signalling proteins (receptors, ion channels, enzymes and transcription factors) that either have transition metal prosthetic groups or thiol/tyrosine residues where NO can exert its effect [52, 53]. One such protein that NO activates by S-nitrosylation is $p21^{ras}$ [53]. This leads to the induction of MAP kinase cascades which can induce apoptosis [33]. NO has been shown to indirectly modify the MAP kinase activity in mammalian tumor cells and neurons [39, 62]. It is important to remark that NO can also inhibit apoptosis [28].

Recent evidence suggests that NO-induced cGMP synthesis is required for NO-induced cell death of cultured *Arabidopsis* cells through the activation of a MAP-kinase upon incubation with different concentrations of NO-donor compounds: sodium nitroprusside (SNP) or Roussin's black salt (RBS) [13]. Apoptotic cell death was also shown to be induced by NO in *Taxus* callus cultures [43].

In plants, the cGMP levels showed a transient increase not only upon addition of NO, but also following gibberelic acid and light stimulation in barley aleurone, bean cells and *Pinus* needles [60]. Similarly, the cGMP levels of tobacco increased when cells were treated with NO [18].

NO, similarly to its activatory role in mammalian defense responses [47, 52], is an important component of the plant disease resistance system [14]. Application of NO donor compounds to or overexpression of recombinant mammalian NOS in tobacco plants or cell suspensions induced the expression of the defence genes encoding the pathogenesis-related-1 (PR-1) protein and the enzyme phenylalanine-ammonia lyase (PAL). Furthermore, these genes proved to be inducible by cGMP-analogues, too. The induction of PR-1 and PAL was also observed partly as a consequence of the increasing cyclic adenosine diphosphate-ribose (cADPR) levels either directly (through S-nitrosylation) or indirectly (in a cGMP-dependent way) induced by NO. Consequently, cGMP and cADPR are second messengers of NO in plants, and they can act synergistically [29], just as reported for gene activation in animal cells [36].

Hypersensitive reaction (HR) is the process of necrotic lesion formation at the site of pathogen entry in order to prevent the pathogen from spreading to uninfected tissues. The first step of the plant hypersensitive reaction is an oxidative burst when so called reactive oxygen species (ROS) like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are generated upon pathogen infection. This burst leads to several effects including the cross-linking of the cell wall [10] and the induction of various plant genes (such as glutathione S-transferase and glutathione peroxidase) involved in cellular protection and defence including the above-mentioned PR-1 and PAL [27,

35]. Salicylic acid (SA) is a signalling molecule that contributes to H_2O_2 production during HR, but high ROS levels can also stimulate SA-production [11]. Sometimes NO was observed to act synergistically with SA and ROS [29, 52]. In soybean, a huge oxidative burst upon pathogen infection could cause only a weak induction of cell death, but the addition of NO significantly increased the strength of the response [14]. The relationship between NO, SA and ROS has been described recently as a self-amplifying process during which redox signalling through NO and ROS can be enhanced by salicylic acid [57]. However, NO can also act independently of ROS to induce the expression of defence-related genes in the case of *Arabidopsis* cell suspension culture [13] and tobacco [18]. The relationship of other signalling molecules (e.g. ethylene, jasmonic acid) in HR to the above-mentioned ones is still being studied intensively.

Furthermore, the oxidative burst has a direct effect leading to host cell death through the Fenton reaction which results the formation of highly reactive species from the less reactive ones [32, 35]. Fenton (or Haber-Weiss) reaction occurs in the presence of free iron in the cytoplasm. The more free iron is present, the more lethal the reaction is. The mRNA binding protein IRP-1 is known to increase intracellular free iron levels in animals by binding to ferritin mRNA and preventing it from translation. IRP-1 is generated from the enzyme aconitase, the activity of which is previously inhibited by binding NO [24]. Plant aconitases have high homologies to human IRP-1 protein, and their activities were also inhibited by NO, suggesting contribution to the defence mechanism against pathogens [41].

Interaction between phytoglobins and NO in plants

The above results gave basis to assume that the interaction between hemoglobins and NO is possible not only in unicellulars and animals, but also in plants. Recent results seem to support this hypothesis.

During hypoxia NO production was observed in maize cell cultures and alfalfa root cultures. Similarly to the earlier described situation in bacteria, phytoglobins could help detoxify this compound by transforming it to nitrate (NO_3^-), which is less toxic for plants. This hypothesis was supported by results showing a greater amount of NO in transformed lines with reduced phytoglobin expression than in wild type or phytoglobin-overproducing lines [16].

Transgenic tobacco seedlings overexpressing alfalfa phytoglobin Mhb1 [49] were shown to grow less slowly upon treatment with NO-generating compound (SNP) than non-transformed seedlings. Furthermore, adult leaves of Mhb1-expressing plants showed a lower extent of necrosis than non-transformed control after treating with SNP. Both findings are assumed to be the consequence of lower intracellular NO levels caused by NO-phytoglobin interaction.

Infection of adult Mhb1-expressing tobacco with the bacteria *Pseudomonas syringae* (Psm) or with Tobacco Necrosis Virus (TNV) also caused a lower extent of leaf necrosis on transformant plants compared to non-transformed control. In the

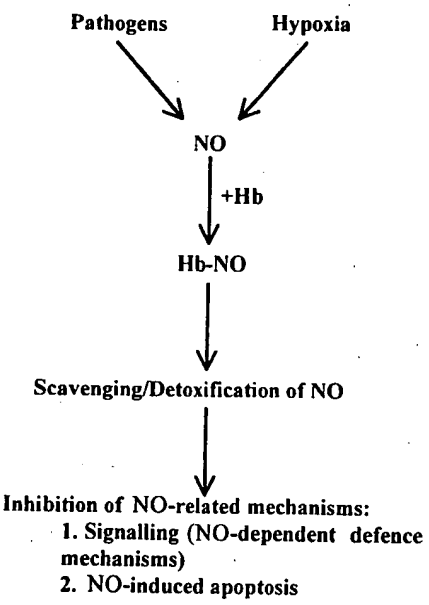

Fig. 2. The possible effect of NO-phytoglobin interaction on NO-dependent mechanisms

transformants, before and after infection with Psm, higher superoxide (O^-) and salicylic acid (SA) levels were measured than in Psm-infected non-transformed plants [49]. It is hypothesized that the lower extent of necrosis on transformant leaves is also the result of intracellular NO level decrease caused by NO-phytoglobin interaction. However, the lower extent of necrosis may not necessarily mean increased resistance of the Mhb1-expressing tobaccos even in spite of their higher ROS and SA levels. This is possible because of the lack of the earlier described synergistic effect [14] between ROS and NO, which cannot be fully compensated by increasing the level of other components in the defence system, e.g. ROS and SA. Figure 2 summarizes how NO-involving processes can be affected by NO-phytoglobin interaction.

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Biologica Hungarica

VOLUME 54, NUMBER 1, 2003

Akadémiai Kiadó, Budapest
MEMBER OF WOLTERS KLUWER GROUP

NO-degradation by alfalfa class 1 hemoglobin (Mhb1): a possible link to *PR-1a* gene expression in Mhb1-overproducing tobacco plants

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Keywords: class 1 hemoglobin, nitric oxide, purification, hexacoordinate, pathogen, *PR-1a*

Abstract

Tobacco plants overproducing alfalfa class 1 hemoglobin (HOT plants) have been shown to have reduced necrotic symptom development. Here we show that this altered pathogenic response is linked to a significant increase in the NO-affected pathogenesis-related (*PR-1a*) transcript accumulation in the transgenic plants. Homogenates of HOT transgenic seedlings were also found to have higher NO-scavenging activity than non-transformed ones. The NO-scavenging properties of recombinant alfalfa class1 hemoglobin have been examined. Recombinant Mhb1 (rMhb1) was produced in bacteria and purified using polyethylene glycol (10-25%) fractionation, chromatography on DEAE-Sephacel, and Phenyl Superose columns. After the final purification step, the obtained preparations were near homogeneous and had a molecular weight of 44 kDa determined by size-exclusion chromatography and 23 kDa by SDS-PAGE indicating that rMhb1 is a dimer. The protein participated in NO-degradation activity with NAD(P)H as a cofactor. After ion-exchange columns, addition of FAD was necessary for exhibiting maximal NO-degradation activity. The NAD(P)H-dependent NO-scavenging activity of rMhb1, which is similar to that of barley hemoglobin, supports a conclusion that both monocot and dicot class 1 hemoglobins can affect cellular NO levels by scavenging NO formed during hypoxia, pathogen attack and other stresses.

Introduction

Nitric oxide (NO) is an important factor in the response to pathogen infection [1, 2]. The response is believed to be through a cGMP-mediated induction of early and late defense reactions. During this event, NO induces guanylate cyclase and the cGMP produced can increase salicylic acid (SA) formation via Ca^{2+}-mediated signalling, resulting in the activation of such defense genes as phenylalanine-ammonia lyase (*PAL*) or pathogenesis-related 1 (*PR-1a*) [1]. The burst of reactive oxygen species (ROS) that occurs after pathogen attack during the hypersensitive response (HR) [3] is also known to induce SA production [4] suggesting that ROS can trigger *PR-1a* expression via an effect on SA production.

Recent findings support the hypothesis that plant (class 1) hemoglobins can interfere with NO-related processes. In tobacco plants, overproduced alfalfa class 1 hemoglobin (Mhb1) can reduce necrotic symptoms compared to nontransformed control after either treatment with an NO-donor sodium nitroprusside (SNP) or infection with viral and bacterial pathogens [5]. Alfalfa root cultures overexpressing barley hemoglobin accumulate less NO than either control or antisense barley hemoglobin alfalfa lines during hypoxic treatment [6]. Cultured alfalfa roots expressing sense or antisense barley hemoglobin have been shown to scavenge NO by converting it to nitrate [7]. This resembles an NO dioxygenase-like activity, earlier reported in the case of microorganisms. The microbial dioxygenase enzyme is a flavohemoglobin with NAD(P)H-dependent enzymatic activity. This enzyme transforms NO to NO_3^- while oxidizing heme, which is then reduced using NAD(P)H [8, 9]. In flavohemoglobins, the globin domain is connected with detoxification of NO, and the flavin domain participates in the reduction of heme from ferric (Fe^{3+}) to ferrous (Fe^{2+}) state. As it has been shown that different hemoglobins can bind and scavenge NO [10, 11], the influence of class 1 hemoglobins on NO-metabolism was attributed to an NO-hemoglobin interaction.

Class 1 hemoglobins are known to possess ligand binding characteristics different from their symbiotic counterparts. While symbiotic hemoglobins have lower oxygen affinity and fast oxygen dissociation rates to facilitate O_2 diffusion [12-14], class 1 hemoglobins have high oxygen affinities and low oxygen dissociation rate constants [15-17]. This implies that class 1 hemoglobins may have functions other than transport of O_2. This difference in ligand binding originates from the difference in the heme coordination of these proteins [15, 18, 19]. Class 1 hemoglobins proved to be hexacoordinate (as opposed to pentacoordinate), referring to the number of bound heme coordination sites in the absence of oxygen. Such hexacoordinate hemoglobins exist throughout the animal and plant kingdoms, in *Synechocystis* [20], *Chlamydomonas* [21], *Drosophila* [22] and in humans (cytoglobin [23] and neuroglobin [24]), but their function is still unclear.

Here we demonstrate that NO-degradation activity is partially retained during the purification of recombinant alfalfa class 1 hemoglobin [25], and this activity is NAD(P)H- and FAD- dependent. This can correspond to earlier suggestions about plant class 1 hemoglobin-NO interaction [26, 27], and may imply a possible role of hemoglobin in NO-involved signalling mechanisms, such as pathogen infection. We also present that NO degradation function was significantly higher in homogenates of transgenic tobacco seedlings previously shown to overexpress Mhb1 compared to nontransformed control. We report that the induction of the pathogenesis related gene

PR-1a was found to be elevated upon pathogen attack in tobacco plants overproducing Mhb1 protein. A potential link of this response to the earlier shown increase in other NO-related signalling components such as ROS and SA [5], is also discussed .

Materials and methods

1. Plant material and homogenization

Tobacco plants (*Nicotiana tabacum* cv. Petit Havanna line SR1) were used to obtain lines overproducing Mhb1 protein (HOT plants) as described in [5]. Two month old SR1 and HOT plants were infected with *Pseudomonas syringae* pv. *maculicola* at a concentration of 10^8 cfu/ml as described in [5]. Homogenates of control SR1 and HOT plants were prepared from 8 day old seedlings by grinding them under liquid N_2 and using 50 mM Tris-HCl pH 7.5 as extraction buffer.

2. Protein production

Mhb1 cDNA was cloned into the BamHI-XhoI restriction sites of the pTRCHis B expression vector (Invitrogen, Carlsbad, USA) polylinker region. The resulting polyhistidine-tagged recombinant Mhb1 protein was 44 amino acids (aa.) longer than its native counterpart. This is partly because of the histidine tag (31 aa.), and partly because of 13 extra amino acids upstream of the Mhb1 protein original start codon (NH_2-DPFVAVNINTLEN-COOH) because of the cloning procedure. This resulted in the ca. 23 kD predicted molecular weight of the recombinant monomer as compared to its original weight of 18 kD based on calculations from the deduced amino acid sequence [25]. Since the additional N-terminal amino acids do not contain any prosthetic groups that could interact with NO, we assume that the observed NO-scavenging originates from the hemoglobin part of the recombinant protein.

3. Extraction and purification of recombinant alfalfa hemoglobin

All procedures were performed at 4 °C, and all chromatographic separations were done on a Pharmacia FPLC protein purification system. All buffers were degassed at 20 °C. Washed cells were resuspended (5g/40ml) in extraction buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 10% (w/v) sucrose, 1 mM EDTA and 20% (v/v) glycerol). Cells were disrupted by two passes through a chilled French pressure cell at approximately 20,000 psi. The lysate was clarified by centrifugation at 27,000x g for 10 min. The supernatant fluid was then fractionated with 10% and then 25% polyethylene glycol (PEG) 8000.

The red coloured 10-25% PEG pellet was redissolved in 30 ml of buffer A (50 mM Tris-HCl pH 8.5, 1 mM EDTA) containing 10% glycerol, and the clarified solution was applied at a rate of 1 ml/min to a DEAE-Sephacel column preequilibrated with buffer A. After a thorough washing, the protein was eluted with a 100 ml linear gradient of 0-500 mM NaCl in buffer A.

The fractions containing the most red colour were pooled and made to 30% saturation with $(NH_4)_2SO_4$ and dissolved. The sample was then loaded onto a Phenyl Superose column equilibrated with buffer A containing 30% $(NH_4)_2SO_4$. Mhb1 was then eluted at a flow rate of 1 ml/min with a 50 ml linear gradient of 30-0% $(NH_4)_2SO_4$ in buffer A. Fractions were analyzed by absorbance, and those containing the most Hb relative to total protein were pooled and concentrated to a final volume of approx. 200 µl

and buffer exchanged into PBS (40 mM KH_2PO_4/K_2HPO_4 pH 7.0, 150 mM NaCl) using a Centricon 10 concentrator (Millipore, Bedford, USA). The purified hemoglobin was then either immediately used for analysis or stored at –80 °C until needed.

4. Molecular mass determination

The native molecular mass of the recombinant alfalfa hemoglobin was determined by size exclusion chromatography on a Superose 12 column using 50 mM Tris-HCl, pH 9.0, 150 mM KCl as column buffer. Fractions (0.5 ml) were collected with a flow rate of 0.5 ml/min and assayed for A_{280} and A_{412}.

5. Measurement of NO conversion by hemoglobin

NO conversion was measured using an NO electrode (NOMK2, World Precision Instruments, USA) in 2 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 1mg/ml bovine Cu,Zn-superoxide dismutase (Sigma) to prevent formation of peroxynitrite [9] and, after ion-exchange chromatography, 5 μM FAD. For NO production, 1 mM sodium nitroprusside was added with continuous stirring, the vial was illuminated to produce NO, and NO reached a saturating concentration of 1-1.5 μM. The sample (1-10 μl) was added followed by the addition of NAD(P)H (0.1 mM). There was no NO decrease with NAD(P)H in the absence of the sample. While testing diphenylene iodonium (DPI), the sample was preincubated for 10 min with the inhibitor before adding NADH. Total protein was determined by the method of Bradford [28].

For NO conversion by HOT and control (SR1) seedling homogenates, we added 5 μl of homogenate to the buffer used for measuring NO-conversion.

6. Electrophoresis and mass spectrometry

SDS-PAGE electrophoresis has been performed using BioRad Mini- Protean II system with acrylamide concentration of 15%. Proteins were stained with Coomassie brilliant blue R-250 and the band of alfalfa hemoglobin was cut out and used for mass-spectrometry. Coomassie stained band was in-situ digested with modified trypsin as described in [29].

MS-MS analysis of the peptide mixture was performed by MALDI Qq-TOF mass spectrometer (Manitoba/Sciex prototype) [30, 31] at Manitoba Proteomics Centre (Winnipeg, Canada). Tandem MS spectra were analysed by using the software package m/z (Proteometrics Ltd., New York, USA) and Sonar MS-MS (Proteometrics Canada) search engine.

7. Semi-quantitative RT-PCR assay

RNA was isolated with Trizol reagent according to the method of Chomczynski [32] and then treated with DNase according to Promega (Wisconsin, USA) protocol. One μg of RNA from each sample was used for the reverse transcription reaction in 20μl with oligo $(dT)_{18}$ primer and M-MuLV reverse transcriptase. Reaction was performed

according to MBI Fermentas Gmbh (St. Leon-Rot, Germany) protocol for first-strand cDNA synthesis. One and a half microlitres of the reverse transcription reaction (0.075 μg RNA of template) were used for PCR analysis of *PR-1a* or one microliter of the reverse transcription reaction (0.05 μg RNA of template) were used for PCR analysis of *EF1α*. PCR amplifications were done in a 25 μl volume containing 1x PCR reaction buffer with cDNA, 0.4 μM specific primers (5'-CACAATTGCCTTCATTTCTTC-3' 5'-CTAGCACAT CCAACACGAAC-3' */PR-1a/*) or (5'-TCACATCAACATTGTGGTCATTGGC-3' 5'-TTGATCTGGTCAAGAG CCTCAAG-3' */EF1α/*), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, and 1.0 unit of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). The PCR reactions were run in a GeneAmp 2400 PCR System (Perkin-Elmer, California, USA) and the products were analyzed by 0.8% agarose gel elecrophoresis with ethidium bromide staining.

8. Quantitative real-time PCR assay

Amplification, data acquisition, and data analysis were carried out using GeneAmp 5700 Sequence Detection System (Applied Biosystem, California, USA). To amplify plant DNA, the real-time PCR used double-stranded DNA-specific dye SYBER Green I. The reaction mixture contained template DNA, 12.5 μl 2x QuantiTect SYBER Green PCR Master Mix (QIAGEN Gmbh, Hilden, Germany), and 50 nM primers in a final volume of 25 μl. PCR reactions were cycled using the following parameters:15 min. initial denaturation at 95 °C and 35 cycles of 94 °C for 20 sec, 59 °C for 20 sec and 72 °C for 20 sec. Template negative reactions were also included in all amplification experiments.

Results

1. Purification of recombinant alfalfa hemoglobin using FPLC

The purification of recombinant alfalfa hemoglobin is shown in Table 1. The protein was purified to a high final purity (after size exclusion chromatography) as assessed by SDS-PAGE (Figure 1). The protein has a molecular mass of ca. 23 kD determined by SDS-PAGE (Figure 1), which is about 5 kD higher than the molecular weight of the native Mhb1 protein (based on calculations from the deduced amino acid sequence [25]). This corresponds to the expected amino acid sequence of the recombinant hemoglobin, which is 44 aa longer than its native counterpart.

Molecular mass of the recombinant protein was determined to be 44±4 kD by size exclusion chromatography on a Superose 12 column. This suggests that the Mhb1 protein is a homodimer, similarly to other plant class 1 hemoglobins.

2. NO-degrading activities of alfalfa class 1 hemoglobin

Table 1 shows both total and specific NO-scavenging activities of the recombinant alfalfa hemoglobin along with its degree of purification after each step. A rapid loss of total NO-scavenging activity can be observed throughout the purification, yet there is a 4 fold increase in the specific NO-scavenging activity up to the point of purification on the Superose 12 column. After the Phenyl Superose step, the activity to degrade NO was still high in the fraction following the hemoglobin peak while hemoglobin concentration at that point decreased by about 10 fold (Figure 2). The specific activity after the Superose 12 column decreased by about 90% and corresponded to 18.5 nmol/min/mg protein (Table 1). To scavenge NO, addition of either NADH or NADPH was required.

Mass spectrometric analysis of the protein band obtained after purification on the Phenyl Superose column showed that it was highly homologous to the native alfalfa class 1 hemoglobin protein sequence (data not shown).

Activity data for the untransformed bacterial extract (lacking recombinant Mhb1 protein) exhibit a dramatic loss both in total and specific activities of NO-scavenging by the end of the second purification step (PEG fraction, Table 2), even though NADH was added at a final concentration of 0.1 mM.

To regenerate oxyhemoglobin from the methemoglobin form, FAD (at a final concentration of 5 μM) was also added to the NO-scavenging (Figure 2) and regeneration (Figure 3) experiments following purification on DEAE-Sephacel and Phenyl Superose columns. No regeneration of oxyhemoglobin from methemoglobin was observed in the presence of NADH without FAD. Activity in the presence of NADPH instead of NADH was about 50±10% of the original rate. The NO-degradation by Mhb1-containing fractions from the Phenyl Superose column could be completely inhibited by 10 μM diphenylene-iodonium (DPI, data not shown).

Homogenates from Mhb1-transgenic tobacco seedlings (HOT 11 and HOT 13 lines) showed increased rates of NADH-dependent NO-scavenging compared to nontransformed SR1 seedlings (Table 3).

3. RT-PCR and real-time PCR of *PR-1a* gene expression in HOT plants upon bacterial infection

Tobacco lines overexpressing alfalfa class 1 hemoglobin (HOT 1, HOT 11, HOT 13 [5]) were infected with *Pseudomonas syringae* pv. *maculicola* (Psm) suspension at a concentration of 10^8/ml. Figure 4a presents pathogenesis-related (*PR-1a*) gene expression levels quantitated by real time PCR assay before and after inoculation of the bacteria or mock inoculation. The *PR-1a* gene expression level in Mhb1-transgenic and control leaves was very low and only little change was observed before 12 hpi. In contrast, at 18 hpi *PR-1a* level in *Mhb1*-transgenic increased approximately 10-fold (Figure 4a) and is higher as compared to control plants. Finally, at 24 hpi, *PR-1a* concentration in Mhb-transgenic lines was 3-times higher compared to control plants (Figure 4a). The observed difference in expression of *PR-1a* gene was repeated in two independent experiments.

Furthermore, semi-quantitative RT-PCR analysis of *PR-1a* gene expression also shows an increase after infection in leaves of Mhb1- transgenic lines compared to nontransformed SR1 plants (Figure 4b). First transcripts of *PR-1a* gene in *Mhb1*-transgenic HOT 11 lines were detected at 12 hours post infection (hpi), and 6 hpi in HOT13 plants. Before 12 hpi no detectable signal of *PR-1a* gene expression in SR1 plants was present. Differences in *PR-1a* gene expression between HOT and SR1 lines were still detectable up to 24 hpi. A similar time course of *PR-1a* gene expression was observed in other Mhb1-transgenic line (HOT 1 line [5] - data not shown). *PR-1a* gene induction was not observed during mock-infection of transgenic or control plants (Figure 4b).

Discussion

Recombinant alfalfa class 1 hemoglobin was purified close to homogeneity and analyzed for its NO-scavenging characteristics. Tobacco lines overexpressing alfalfa class 1 hemoglobin (HOT lines, [5]) were used to study possible consequences of hemoglobin-NO interaction with respect to responses to pathogen infection.

There is an inverse relationship between the levels of class 1 hemoglobins and NO in tissue during hypoxic stress [6, 33] and there are implications of a relationship between the two compounds in reducing effects of NO or pathogen attack in HOT tobacco plants [5]. Extracts of alfalfa roots overexpressing barley hemoglobin possess an NAD(P)H-dependent NO dioxygenase activity that is dependent on the presence of the hemoglobin [7]. The demonstration that partially purified preparations of rMhb1 possess an NAD(P)H- and FAD-dependent NO-scavenging activity indicate that alfalfa class 1 hemoglobin is also capable of participating in NO turnover. As with the case for barley hemoglobin, the high NO-scavenging activity at decreasing hemoglobin concentrations after the Phenyl Superose step (Figure 2), and the loss of specific activity after the size-exclusion (Superose 12) step indicate that some other protein likely possessing methemoglobin reductase activity is a necessary component in NO-scavenging together with the alfalfa recombinant hemoglobin.

In the case of *Vitreoscilla* hemoglobin (*Vhb*), which is capable of acting either as a single domain homodimer or as a two-domain heterodimer *in vivo*, NO-scavenging ability was reported to improve significantly upon its association with the flavoreductase domain of *Ralstonia eutropha* flavohemoglobin [34]. However, unlike flavohemoglobins, class 1 hemoglobins do not possess a flavin domain. Furthermore, a loss of bound FAD was observed during flavohemoglobin purification, so external FAD was required to recover the NO-scavenging activity [8]. This may be similar to the observation that addition of external FAD was necessary during purification of the recombinant Mhb1 protein probably to preserve the methemoglobin reductase activity of the above mentioned hypothetical flavoprotein.

Similarly to mammalian systems [10, 11], a functional hemoglobin-NO interaction *in planta* may also reduce intracellular NO levels and affect a variety of NO-related processes (reviewed in [35-37]). *Pseudomonas syringae* pv. *glycinea* is known to induce NO-production upon infection [38]. In the case of pathogen infection, the supposed lower levels of NO in Mhb1-overproducing plants could cause a fundamental change throughout the NO-mediated signalling cascade. A balance between NO and H_2O_2 is thought to be necessary for initiating the process of hypersensitive cell death [39]. In HOT plants, the increased levels of NO resulting from the infection process would be expected to be reduced by the reaction with the over-expressed class 1 hemoglobin, disrupting the hypersensitive cell death response and resulting in a smaller necrotic spot, in line with the observed result [5].

In contrast to programmed cell death, the induction of pathogenesis related genes is known to be a late defense response. The interrelationships between NO, ROS, SA and late defense-response, such as *PR-1a* gene induction, have been well documented [40]. An NO-dependent and a ROS-dependent pathway, both involving SA, have been proposed in the induction of *PR-s*. NO levels in hypoxic plant tissue have been shown to vary independently with Hb [6, 33]. In Psm-infected HOT plants, a significant increase

was detected in *PR-1a* gene induction compared to controls (Figure 4). ROS and SA quantities of Psm-infected HOT lines have been found to be significantly higher compared to an uninfected control [5]. Some points with respect to Hb, NO and ROS are germane to the discussion. Both barley [7] and alfalfa hemoglobin (Table 3) are components of an NO-dioxygenase activity. Bacterial flavohemoglobins, under aerobic conditions, generate superoxide [41], while alfalfa roots overexpressing barley Hb have increased H_2O_2 levels (manuscript in preparation). This cumulative data would suggest that the *PR-1a* response observed in HOT lines occurs via a ROS-SA pathway since the steady state NO levels in infected plants would be expected to be low due to the presence of hemoglobin.

As NO-scavenging activity was not only observed with rMhb1 protein from alfalfa, but also with barley hemoglobin [7], we can assume that these proteins from both monocot and dicot plants may have such a role. Indeed, among other globins and heme proteins, class 1 hemoglobins of different plants share the same hexacoordinated heme structure [15, 16] that results in tight ligand binding [15, 42].

In summary, our results implicate class 1 hemoglobin in NO-scavenging and that this scavenging can affect an NO-regulated process such as the hypersensitive response associated with pathogen infection.

Acknowledgements

The authors are grateful to Dr. Natalia V. Bykova for performing mass-spectrometry measurements and thank Doug Durnin for his excellent technical assistance. A.M. and J.H. were supported by a grants from EC in Centre of Excellence Program (contract no. ICA1-CT-2000-70010) and State Committee for Scientific Research (no.6 P04A 014 20). RDH received support from the Natural Sciences and Engineering Research Council of Canada (RGP4689) and Genome Canada.

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Table 1. Purification of Mhb1 recombinant protein produced by *E. coli*

Fraction	Volume (ml)	Total protein (mg)	Total rMhb1 (mg)	Degree of rMhb1 purification	Total NO-scavenging activity (nmol NO/min)	Specific NO-scavenging activity (nmol NO/min/mg protein)
Total extract	55	764.5	48.93	1	42765	55.94
10-25% PEG redissolved pellet	30	285.3	30.53	1.67	6055	21.22
DEAE-Sephacel	16	28	5.6	3.12	2490	88.93
Phenyl Superose	4	1.564	1.030	10.28	330	211
Superose 12	0.1	0.0938	0.0856	14.26	1.74	18.55

Table 2. NO-scavenging activity in fractions from non-transformed *E. coli*

Fraction	Volume (ml)	Total protein (mg)	Total activity (nmol NO/min)	Specific activity (nmol NO/min/mg protein)
Total extract	22.88	255.3	9848	38.57
10-25% PEG pellet	12	75.48	144	1.90
DEAE Sephacel	4.5	1.66	0	0

Table 3. NO-conversion activities of seedlings

Tobacco lines	Rate of NO-scavenging (μmol NO/min/g FW)	Ratio (%)
SR1	0.173±0.004	100
HOT 11	0. 205±0.010	118
HOT 13	0.274±0.030	158

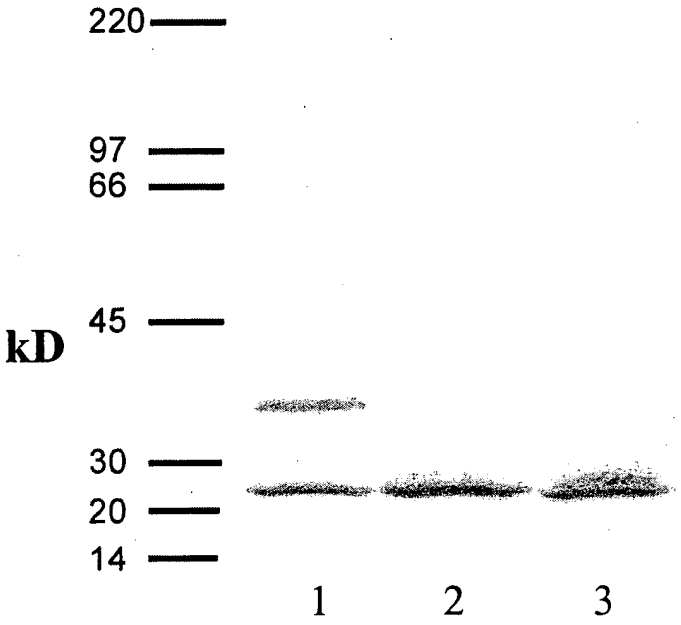


Figure 1

SDS-PAGE showing three stages of purification (each lane contains 8 μg of protein). Lane 1: sample after DEAE-Sephacel column. Lane 2: sample after Phenyl Superose column. Lane 3: Sample after size-exclusion (Superose 12) column

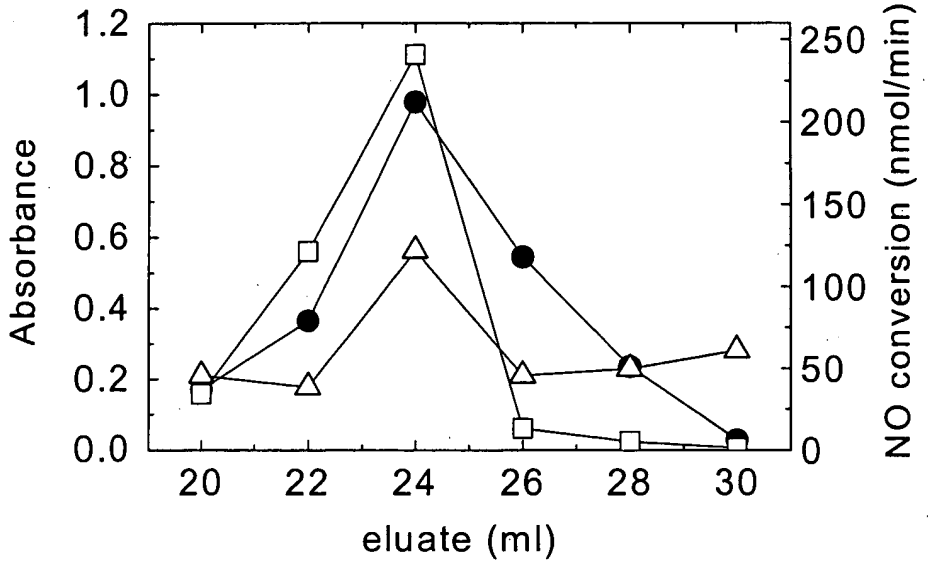


Figure 2

Elution profiles from Phenyl Superose column of total protein (A_{280}, open triangles), hemoglobin (A_{412}, open squares) and NO conversion activity (black circles)

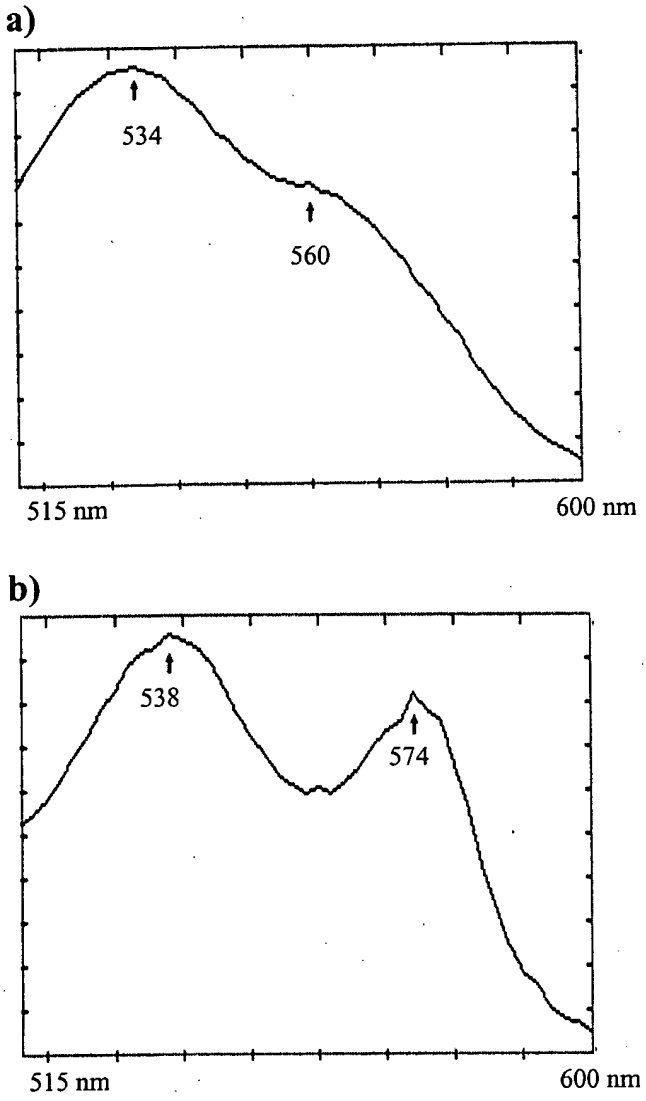
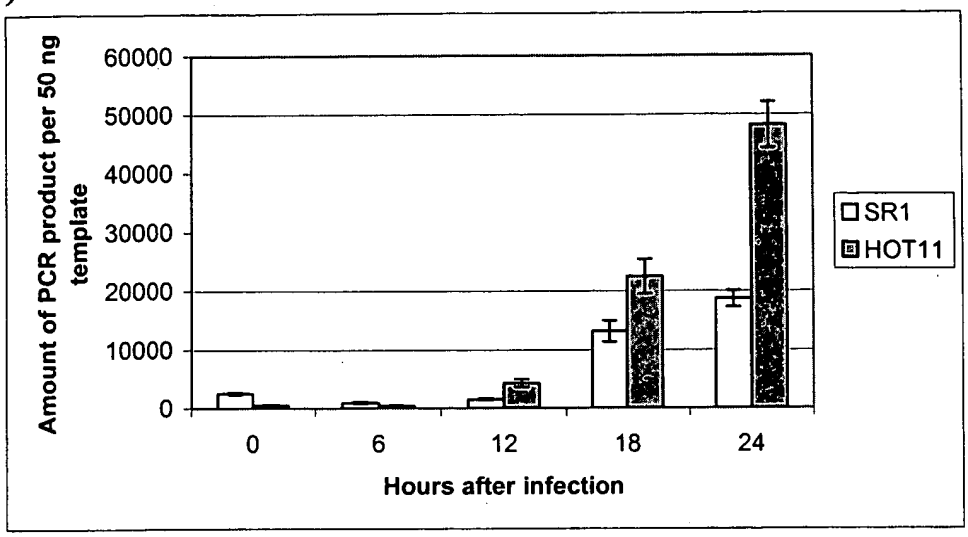


Figure 3

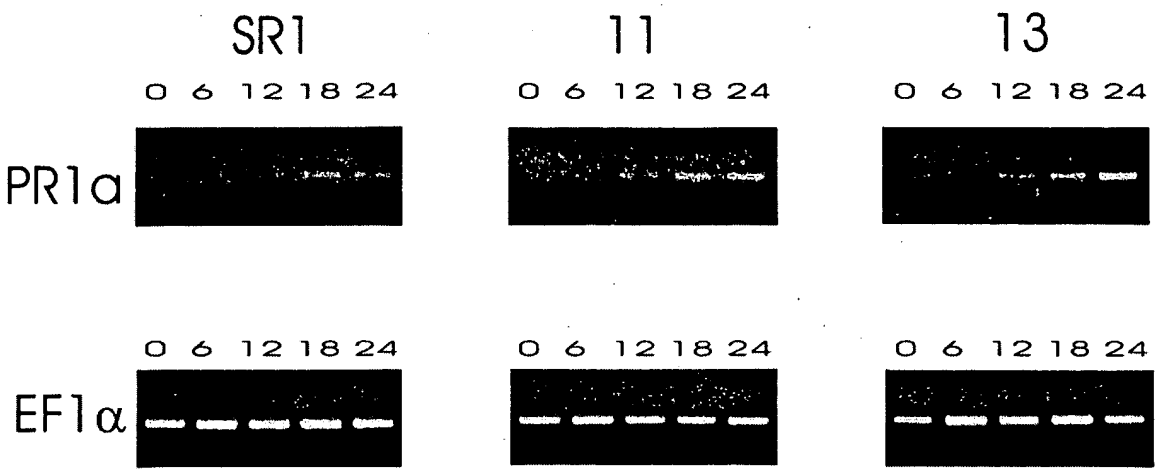
Spectra of purified recombinant alfalfa hemoglobin. a: ferric methemoglobin form, b: formation of oxyhemoglobin after incubation with 0.1 mM NADH and 5 μM FAD.

a)


Amount of PCR product per 50 ng template
60000
50000
40000
30000
20000
10000
0
0
6
12
18
24
Hours after infection
SR1
HOT11

b)

Pseudomonas syringae pv *maculicola*



Mock

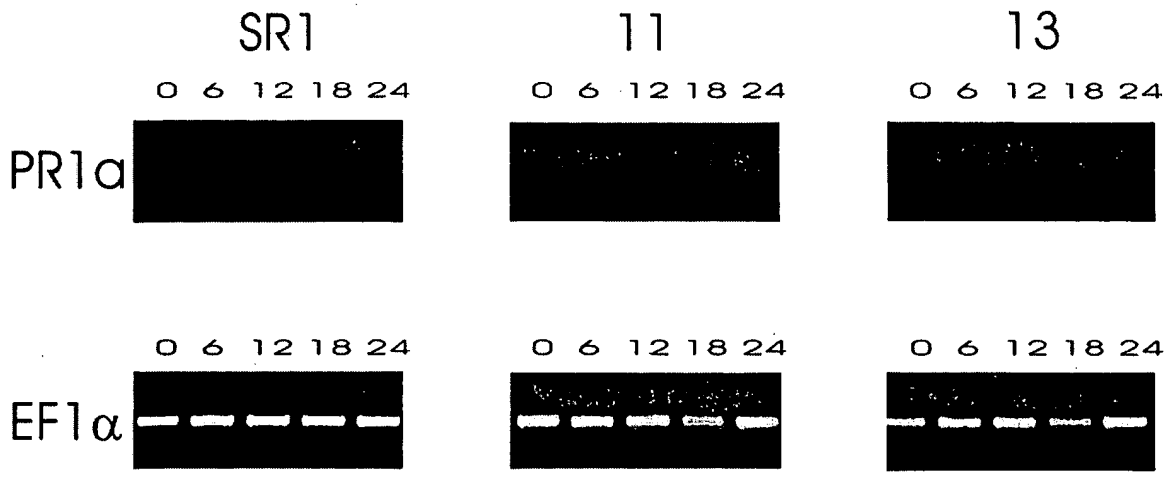


Figure 4

a: Quantitation of change in *PR-1a* expression levels by real time PCR before and upon infection of tobacco leaves (SR1: nontransformed control; HOT11: Mhb1-overporducing plant) with *P. syringae* pv. *maculicola*

b: Time course analysis of *PR-1a* gene expression by RT-PCR before and upon infection of tobacco leaves (SR1: nontransformed control; 11, 13: independent HOT lines) with *P. syringae* pv. *maculicola* (0, 6, 12, 24 hours after infection, respectively). To verify the equal loading of RNA samples, the same reaction was carried out using specific primers of *EF1* α (elongation factor 1 alpha) gene.

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  ORGANISM  Hordeum vulgare
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REFERENCE   1  (bases 1 to 1173)
  AUTHORS   Guy,P.A., Sowa,A. and Hill,R.D.
  TITLE     Genomic nucleotide sequence of the barley hemoglobin gene
            (Accession No. U94968) (PGR 97-093)
  JOURNAL   Plant Physiol. 114, 1135 (1997)
REFERENCE   2  (bases 1 to 1173)
  AUTHORS   Guy,P.A., Sowa,A.W. and Hill,R.D.
  TITLE     Direct Submission
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                     AE"
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